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Cellular Inflammatory Response of *Penaeus aztecus* and *P. setiferus* to the Pathogenic Fungus, *Fusarium* sp., Isolated From the California Brown Shrimp, *P. californiensis*[1]

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Host response of two penaeid species, *Penaeus aztecus* and *P. setiferus*, from the Gulf of Mexico to the pathogenic fungus *Fusarium* sp. isolated from the California brown shrimp, *P. californiensis*, was studied in vivo. The hemocytic response to this fungus was traced histologically in the gills. Both species showed complete resistance to infection by the fungal spores when normal or wounded shrimp were held in seawater containing the spores or when spores were injected directly into the shrimp in low concentrations. Complete melanization and encapsulation of the micro- and macroconidia were observed. Spore dosages of 3.2×10^6 or more were lethal, apparently due to mechanical blockage of the blood sinuses of the gills.

INTRODUCTION

Large-scale mortalities and prevalence of infection of nearly 100% due to a *Fusarium* sp. were reported from populations of raceway-reared California brown shrimp, *P. californiensis*, at the University of Sonora–University of Arizona experimental shrimp farm at Puerto Peñasco, Sonora, Mexico (Lightner, 1975). The primary lesion seen in affected shrimp during the epizootic was caused by the invasion by the fungus of the basal segment of the pereiopods and adjacent portions of the body wall and the invasion of the gills. Except in moribund shrimps, all of the sites of infection were blackened. These blackened lesions were shown to be the result of an intense hemocytic response to the fungus in the tissues that resulted in encapsulation and subsequent melanization of hyphal elements (Lightner, 1975).

Similar *Fusarium* spp. have been described from other penaeid species and from a lobster. A seemingly different species of *Fusarium* than the one found in *P. californiensis* was described from *P. japonicus* by Egusa and Ueda (1972). Serious mortalities occurred in pond-cultured populations of *P. japonicus* as a result of infection by this fungus. The disease was characterized by the presence of many black spots in the gills.

Johnson (1974) observed a *Fusarium* sp. in the gills of laboratory-held pink shrimp, *P. duorarum*, in Texas but did not observe mortalities due to the infection. Johnson did note involvement of the gills in affected animals. Lightner and Fontaine (1975) reported mortalities of 35% due to a *Fusarium* sp. in cultured American lobsters, *Homarus americanus*. The first sign of disease in infected lobsters was the appearance of white spots on the exoskeleton 6 to 10 days after a normal molt. These white spots ultimately turned black. Affected lobsters did not survive the next molt. Examination of the gills of dead or moribund lobsters revealed nearly complete destruction of the gills by the expanding mycelium.

The present study was planned to follow the course of infection by the *Fusarium* sp. from *P. californiensis* in *P. aztecus* and *P. setiferus* after addition of conidia to the water and after injection of conidia directly

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into the hemocoel of the shrimp. To our surprise, patent infections like those seen in *P. californiensis* could not be established in *P. aztecus* or *P. setiferus*, except when high dosages of conidia (over 10^6 conidia per animal) were injected. Hence a second study was conducted to determine the fate of injected conidia in these apparently resistant species.

MATERIALS AND METHODS

The brown shrimp, *Penaeus aztecus*, used in this study were hatched from spawn from wild female shrimp taken from the Gulf of Mexico. Larval shrimp were reared at the Galveston Laboratory according to the methods of Mock et al. (1973). The average total length of brown shrimp used was 111 mm. The white shrimp, *P. setiferus*, used in this study were purchased live from a commercial bait dealer on West Galveston Bay, Texas. These shrimp averaged 115 mm in total length and were acclimated to laboratory conditions 28 $^0/_{00}$ salinity) for 48 hr in a 600-liter fiberglass tank.

Six glass aquaria used for the experiment were filled with 60 liters of seawater each and provided with two air-lift operated, crushed oyster shell filters. The bottoms of the aquaria were lined with a 1-cm layer of fine sand. Water temperature varied between 23° and 26° C, and salinities varied between 26 and 28 $^0/_{00}$ during the study.

The *Fusarium* sp. isolated from the California brown shrimp, *P. californiensis*, was cultured on Sabouraud's dextrose agar medium (Difco[4]) supplemented with 2% NaCl and shrimp extract (Lightner and Fontaine, 1973). Cultures were incubated at 28° C. Large numbers of macroconidia (3×10^9 average per culture) were obtained in saline washings of the agar surface from a single 100-mm diameter culture dish after 10 days incubation. The macroconidia were three to four-celled, canoe-shaped, and ranged from 30 to 48 μm in total length. All spore counts were made using a Bright Line[4] hemocytometer.

[4]The use of trade names in this publication does not imply endorsement of commercial products.

Three experiments were carried out. The purpose of Experiment 1 was to determine if brown and white shrimp could be infected by the *Fusarium* sp. from *P. californiensis*. Experiment 2 was run to estimate the minimum number of macroconidia of the *Fusarium* sp. needed to kill brown and white shrimp challenged by injection within 3 to 5 days. In Experiment 3 the hemocytic response of brown and white shrimp to injected macroconidia of the *Fusarium* sp. was followed microscopically.

Experiment 1. In this experiment three groups each consisting of five brown and five white shrimp were exposed to conidia of *Fusarium* sp. by three different methods.

The first group of five brown and five white shrimp were exposed to the fungus in a 60-liter glass aquarium to which 25 ml of a saline suspension containing 1.5×10^9 macroconidia was added.

A second group in a similar aquarium was exposed to the fungus by addition of 25 ml of the same suspension of macroconidia, but the animals in this group were wounded immediately prior to exposure. Shrimp were wounded by unilateral excision of a piece (approximately 3 by 10 mm) of the lower portion of the branchiostegal carapace and by unilateral amputation of one of the 5th pereiopods through the coxal segment.

A third group of five brown and five white shrimp were exposed to the *Fusarium* sp. by injection of 0.1 ml of saline suspension containing 3.2×10^7 macroconidia per milliliter into the hemocoel at the base of the 5th pereiopod.

Three control aquaria, each with five brown and five white shrimp, were also prepared. The first control aquarium received no treatment. In the second control aquarium, the shrimp were wounded as described previously, and shrimp in the third control aquarium were injected with 0.1 ml of sterile saline at the base of the 5th pereiopod. Both control and experimental groups were fed a pelleted ration (Arizona Maintenance diet, Arizona Feeds, Tucson, Arizona) daily during the 10-day period of the study.

Experiment 2. The minimum number of

TABLE 1
Results of Experiment 2 in Which Brown (*Penaeus aztecus*) and White (*Penaeus setiferus*) Shrimp were Injected with Various Concentrations of a *Fusarium* sp.

Number of spores per 0.1 ml	Total number of shrimp	First mortality (hr)	Survival after 5 days
Brown, *P. aztecus*			
24	5	24	4
240	5	24	4
240, 000	5	12	2[a]
600, 000	5	8	2
900, 000	5	6	0[b]
White, *P. setiferus*			
24	5	–	5
240	5	24	4
240, 000	5	36	3
600, 000	5	12	2
900, 000	5	12	1[c]

[a] One apparent death (trapped in the air-lift pipe).
[b] After 72 h.
[c] Died on the sixth day.

macroconidia causing death within a 5-day period was approximated. Macroconidia were harvested as described previously from 10-day-old cultures grown on Sabouraud's dextrose agar. The following suspensions of macroconidia were prepared: 2.4×10^2, 2.4×10^3, 2.4×10^6, 6.0×10^6, and 9.0×10^6 macroconidia/ml of saline. Five different groups of five brown and five white shrimp each were injected with 0.1 ml of each of the five conidiospore suspensions (Table 1). Group 1 received 0.1 ml of the suspension containing 2.4×10^2 macroconidia or about 24 macroconidia per shrimp; group 2 shrimp received approximately 240 macroconidia per shrimp, etc.

Experiment 3. Fifteen brown and 15 white shrimp were injected at the base of the 5th pereiopod with 0.1 ml of a saline suspension containing approximately 8×10^6 macroconidia/ml, or about 8×10^5 macroconidia per shrimp. A control group received 0.1 ml of sterile saline by injection at the base of the 5th pereiopod. Two shrimp of each species were taken at 0, 4, 8, 12, 24, 28, and 48 hr after injection for microscopical examination. One of the specimens of each species was preserved for histological examination, and the other was used fresh for preparation of wet mounts of the gills. The 0-hr shrimp served as normal control animals and were not injected with conidiospores. Wet mounts of one or two gill processes were prepared and examined microscopically in seawater at 100X. Samples for histological examination were fixed for 24–48 hr in Davidson's fixative and then embedded in Paraplast Plus (Curtin Scientific Company) using standard dehydrating and embedding procedures. Cross sections, 8 to 10 μm, were made through the cephalothorax to include the bases of the 5th pereiopods. The site of injection and portions of the gills, hepatopancreas, and heart were typically present in these sections. Histological sections were stained either with hematoxylin and eosin or periodic acid-Schiff (PAS).

RESULTS

Experiment 1. No mortalities occurred among shrimp exposed to water with conidia or in the control group during the 10-day period following inoculation of the experimental aquarium. Examination of wet mounts of gill processes prepared from the gills of all 10 experimental shrimp and three control shrimp at the termination of the experiment revealed no conidia, hyphae, or other signs of infection.

Among wounded shrimp exposed to conidia two mortalities occurred, and one occurred in the control group during the study, but no conidia, hyphae, or other aparent signs of a *Fusarium* infection were observed in mounts prepared from the gills of these shrimp. Furthermore, examination of wet mounts of the gills of the remaining eight experimental shrimp at the termination of the experiment did not reveal conidia, hyphae, or other signs of infection.

All five brown shrimp injected with a 0.1-ml suspension of 3.2×10^6 macroconidia died within 16 hr post-injection. The five white shrimp given the same injection of conidia all died within 24 hr post-injection. Wet mounts prepared from the gills of these shrimp showed macroconidia and hyphae

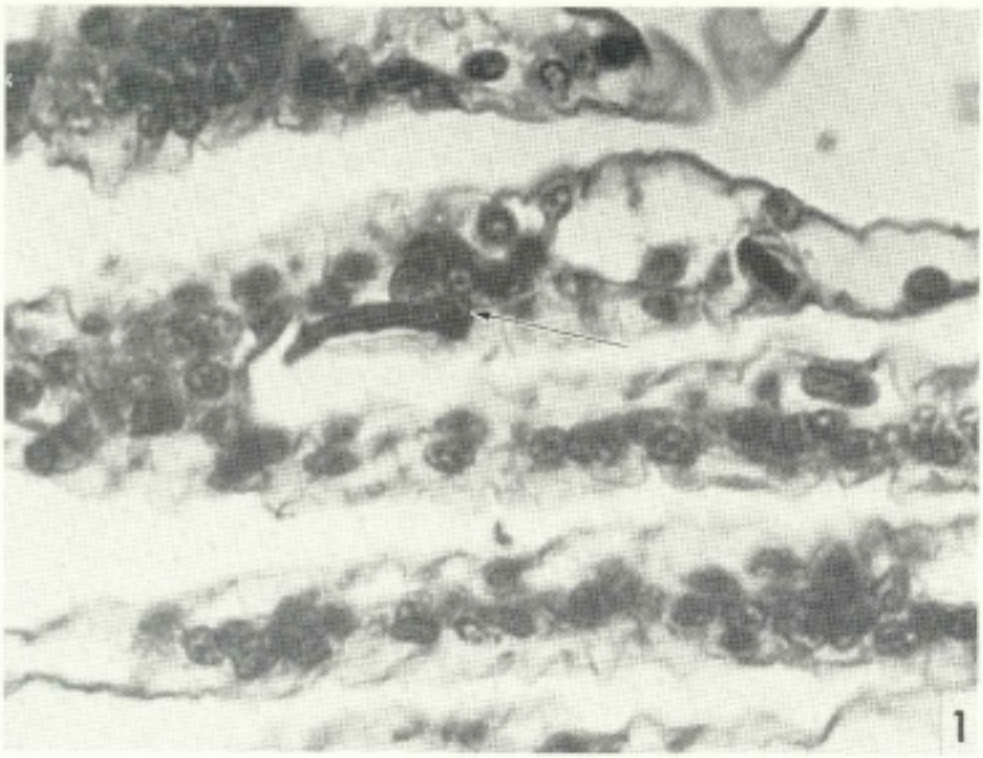

FIG. 1. A single macroconidium within a gill lamella of a white shrimp (*Penaeus setiferus*) 4 hr after injection. Hemocytes are attached to one of the terminal cells of the conidiospore (arrow). PAS stain. × 880.

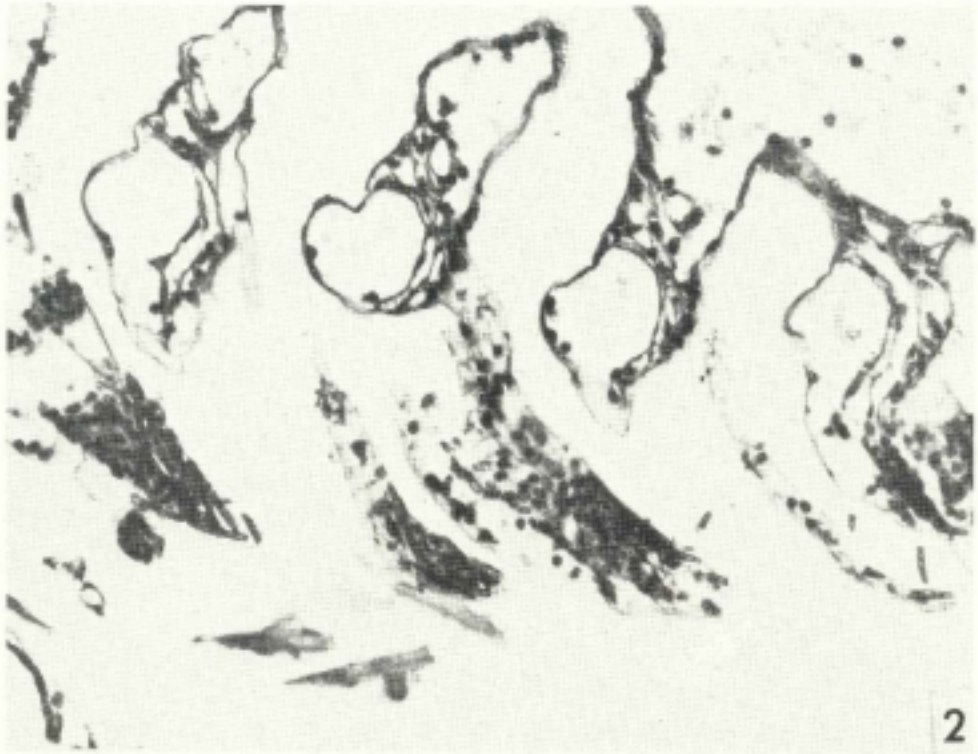

FIG. 2. Photomicrograph of gill lamellae from a brown shrimp (*Penaeus aztecus*) 8 hr after injection. Aggregates of macroconidia are present in the distal portion of the gill lamellae. A slight hemocytic response is apparent. PAS stain. × 200.

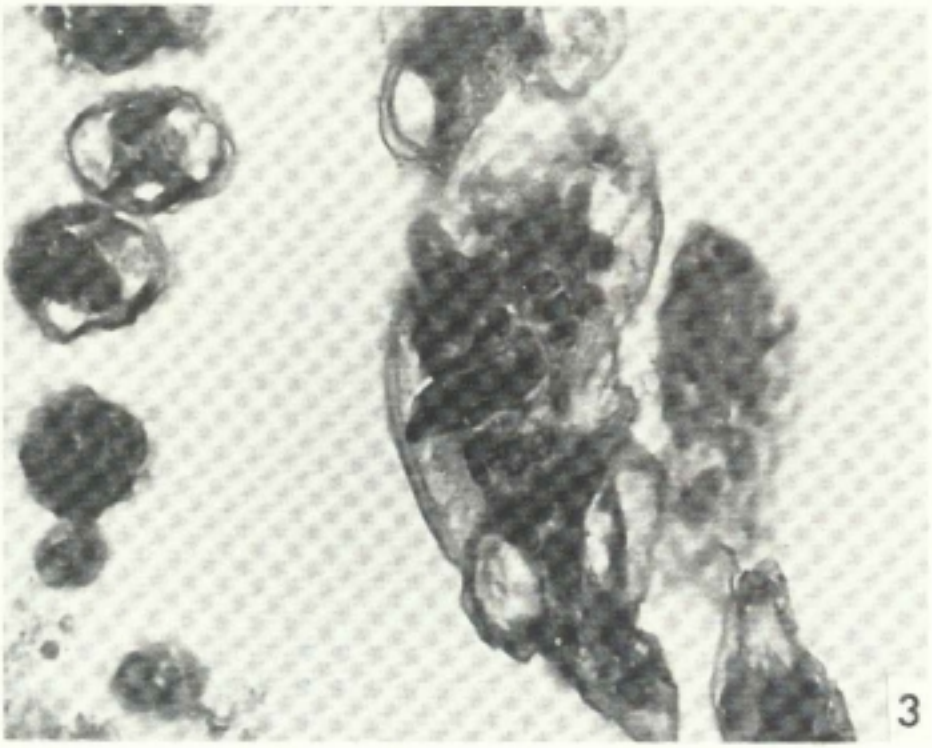

FIG. 3. Macroconidium within a gill lamella of a white shrimp (*Penaeus setiferus*) is shown encapsulated by a single layer of hemocytes 24 hr after injection. PAS stain. × 880.

literally filling the distal portions of the gill lamellae. Hemocytic response to the conidia and hyphae in the gills was negligible, and melanization was absent. No mortalities occurred in the control group.

Experiment 2. The results of this experiment are tabulated in Table 1. For both brown and white shrimp direct exposure to 9.0×10^4 macroconidia by injection into the hemocoel was lethal to nine of the 10 shrimp within 5 days. On the other extreme, only one brown and no white shrimp died during the experimental period in shrimp receiving approximately 24 macroconidia.

Wet mounts of gills prepared from moribund and dead shrimp revealed the presence of macroconidia and hyphae. However, the gills of shrimp of both species that survived this 5-day period did not contain macroconidia or hyphae at 15 days post-injection. Furthermore, remains of the melanized capsules present at 48 hr post-injection were not observed after 15 days.

Experiment 3. No significant histological differences were observed in the response to injected *Fusarium* macroconidia in brown and white shrimp. In both species injected macroconidia were present only in the gills at 4 hr post-injection, and macroconidia could not be demonstrated at the site of injection, in the hepatopancreas, or in the heart. Macroconidia were accumulated singly and in aggregates in the distal portions of the gill lamellae (Figs. 1, 2). There was a slight response by the hemocytes of both brown and white shrimp by 4 hr post-injection, and a few hemocytes were observed adhered to the macroconidia. Usually hemocytes adhered to the terminal cells of the macroconidia, but some were observed adhered to the center cells (Fig. 1).

Within 24 hr post-injection many of the macroconidia had been encapsulated by a single layer of hemocytes, and others were becoming encapsulated (Fig. 3, 4). Gill lamellae that contained macroconidia were heavily congested with hemocytes, particularly near the distal tips where the macroconidia had accumulated and were being encapsulated. Melanization of conidia or

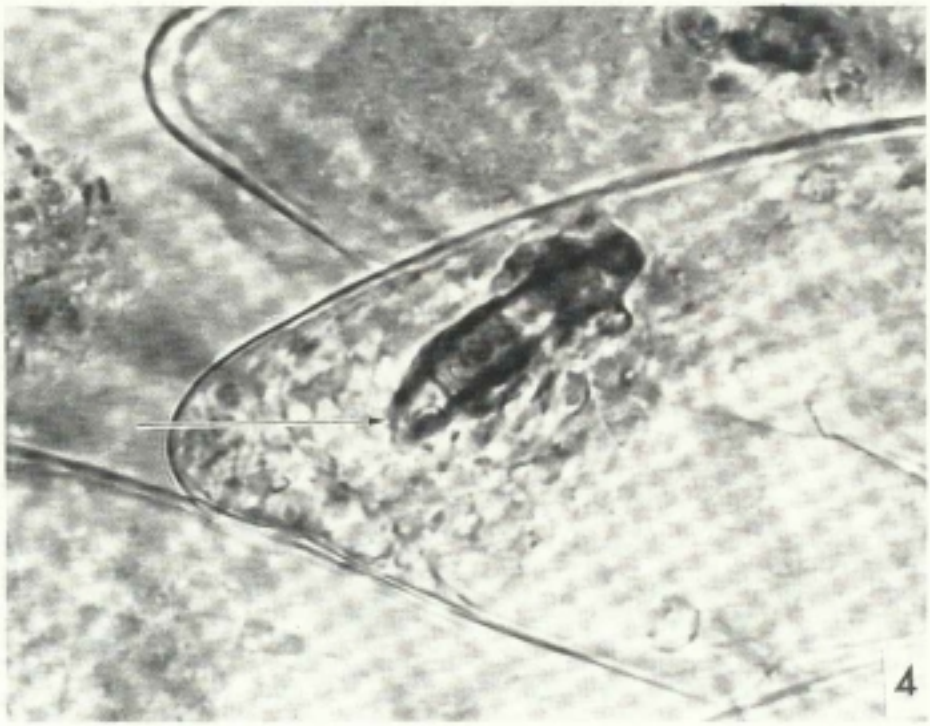

FIG. 4. Wet mount of a gill lamella from a white shrimp (*Penaeus setiferus*) containing a single partially encapsulated macroconidium. Hemocytes have covered all but one of the cells (arrow) of the conidiospore. No stain. × 920.

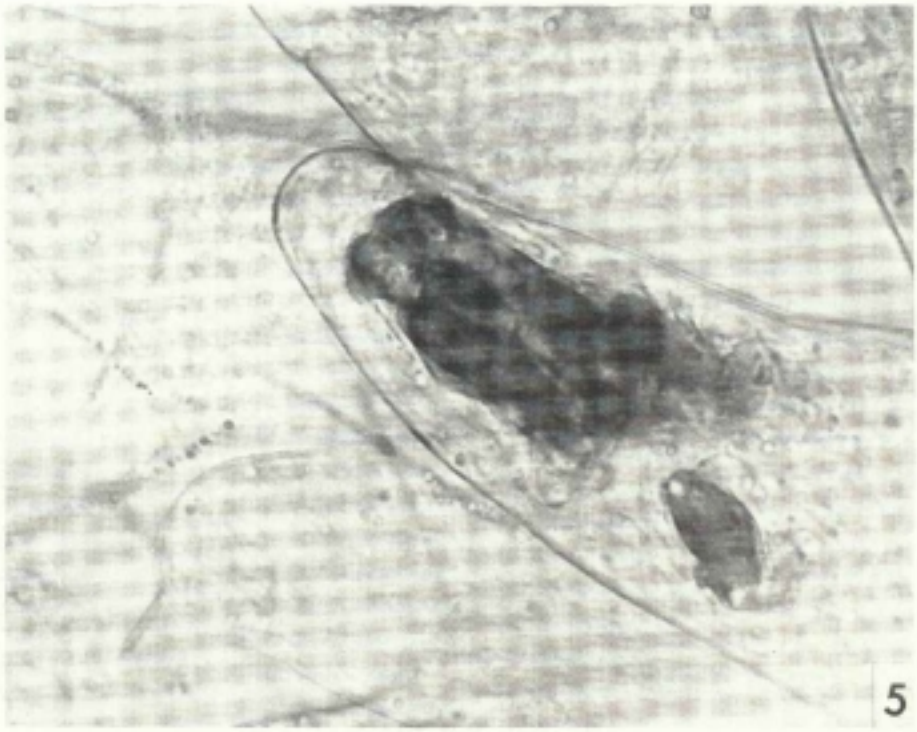

FIG. 5. Fresh gill mounts of brown shrimp (*Penaeus aztecus*) 28 hr after injection. The process of encapsulation and melanization of the macroconidia has been completed, and the conidiospores are being lysed. No stain. × 600.

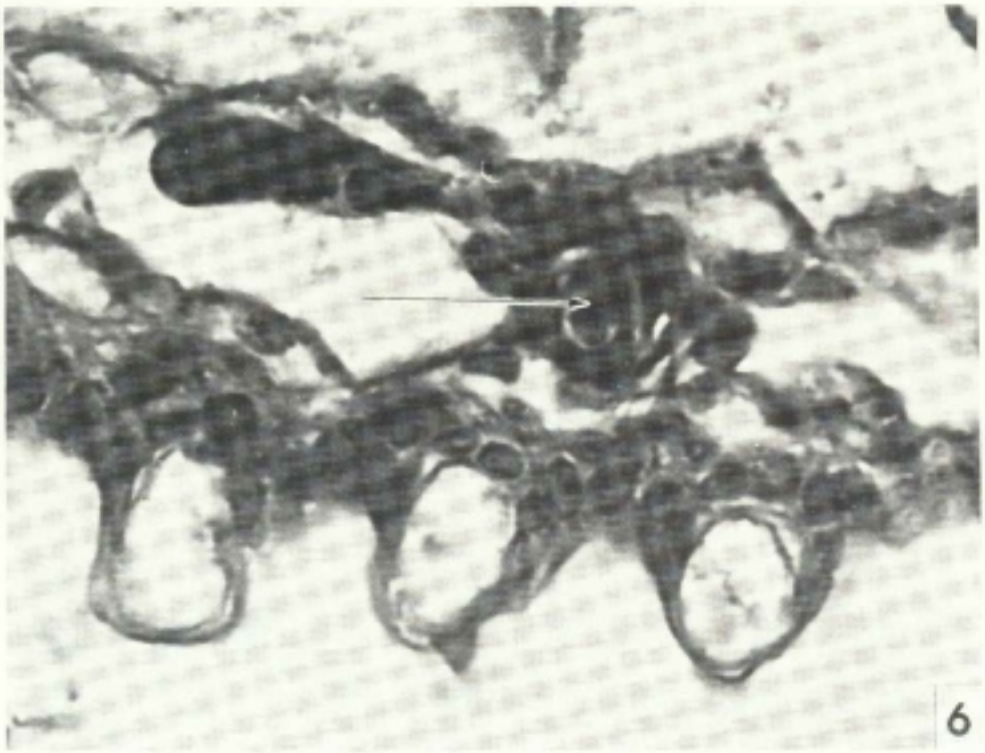

FIG. 6. Cross section through a macroconidium present in the gill lamella of a white shrimp (*Penaeus setiferus*) 28 hr after inoculation. The conidiospore (arrow) is surrounded by multiple layers of hemocytes. PAS stain. × 1170.

hemocyte accumulations was not observed at this time.

Melanization of hemocyte-macroconidia capsules was evident at 28 hr post-injection, with many of the capsules being pale yellow to light brown in color (Fig. 5). Capsules were composed of multiple layers of flattened hemocytes (Fig. 6), and the thick accumulation of hemocytes combined with melanin deposition made observations of conidia within the hemocyte capsules difficult. In wet mounts of the gills, hemocyte-macroconidia capsules were grossly visible as small brown or black specks in the gills.

By 48 hr post-injection most of the hemocyte-macroconidia capsules had become dark brown due to heavy deposition of melanin. The macroconidia themselves were difficult to discern morphologically and were apparently being lysed (Figs. 7, 8). The only evidence of macroconidia within the heavily melanized capsules in the tips of the gill lamellae at 48 hr post-injection was the presence of amorphous masses of PAS-positive debris in the centers of the capsules.

DISCUSSION

The mechanisms of phagocytosis and elimination of foreign and necrotic material have been documented in insects (Werner and Jones, 1969; Salt, 1970; Ryan and Nicholas, 1972) and in the white shrimp (Fontaine and Lightner, 1974). In insects the typical hemocytic response to foreign particulate material was described as phagocytosis, encapsulation, and melanization, the process being dependent on particle size (Salt, 1970). In the study reported by Fontaine and Lightner (1974) carmine particles when injected into the abdominal musculature were phagocytosed by circulating hemocytes and "fixed phagocytes" in the gills and other tissues. By 1 hr post-injection accumulations of hemocytes containing carmine were visible in the gills, and by 30 hr carmine was visible grossly only in the gills, in the heart, and at the site of injection. The heaviest accumulations of carmine throughout the study were in the gills.

In the present study, the gills appeared to

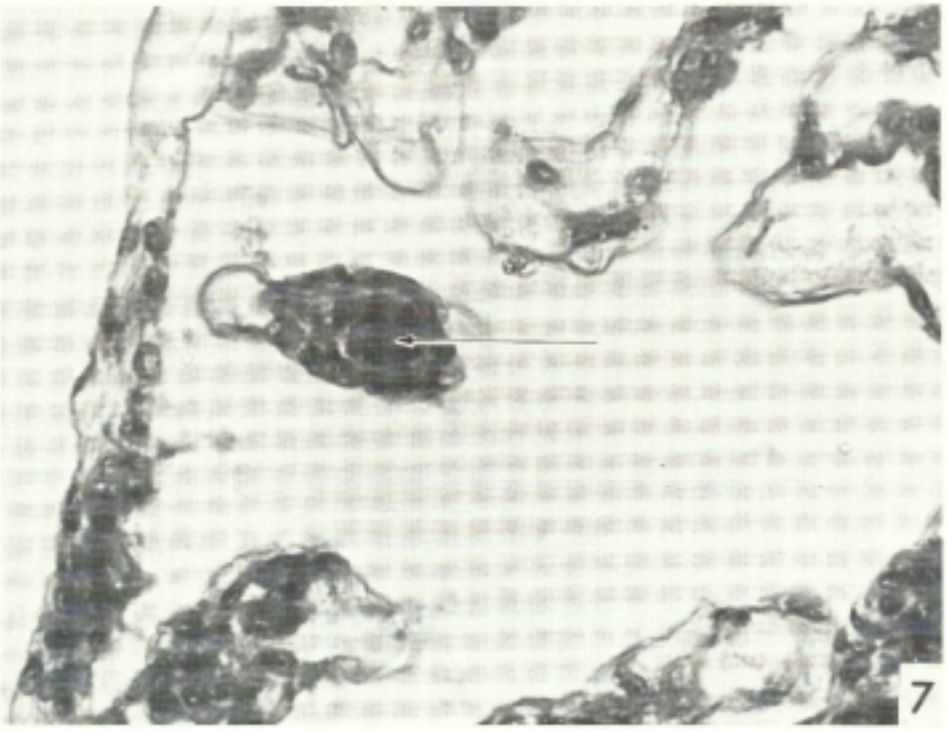

FIG. 7. Photomicrograph of an amorphous mass of PAS-positive debris (arrow) encircled by multiple layers of hemocytes in a cross section of a gill lamella of a brown shrimp (*Penaeus aztecus*) 48 hr after injection. PAS stain. × 700.

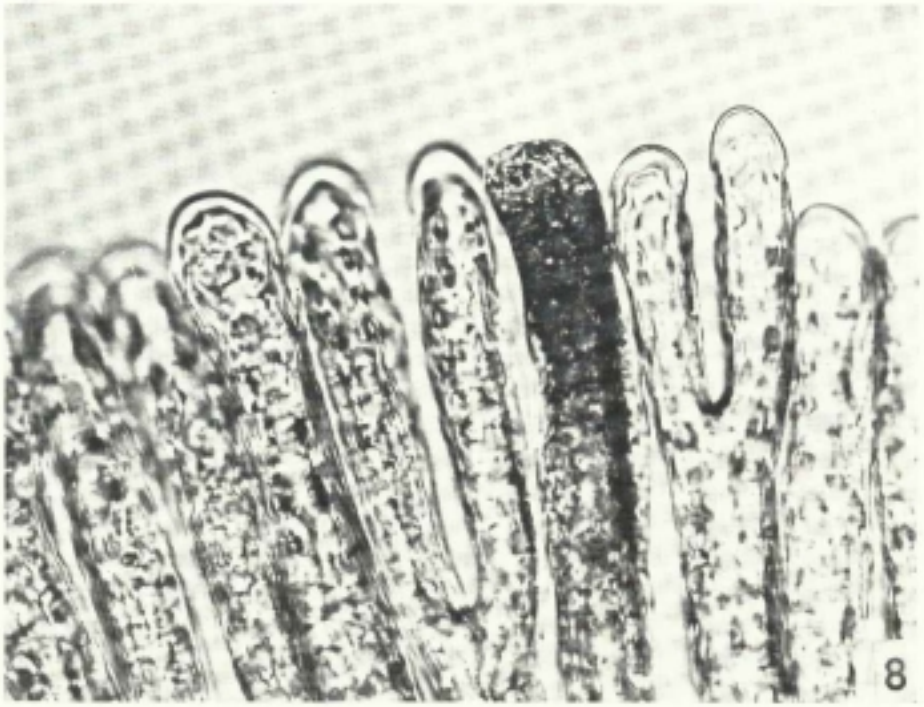

FIG. 8. Photomicrograph of a wet mount of a gill lamella from a white shrimp (*Penaeus setiferus*) at 48 hr after injection. The remains of macroconidia are apparent within the heavily melanized distal portion of the gill filament. No stain. × 370.

be the only organ in which the macroconidia were accumulated and later eliminated. Whether the macroconidia reached the gills by a hemocyte mediated mechanism was not determined. However, once the macroconidia had reached the gills, they were attacked by the hemocytes and eventually destroyed, except when high dosages of conidiospores were administered.

In the first experiment germination of macroconidia and production of vegetative hyphae were observed in dead and moribund animals. Shrimp were given approximately 3.2×10^6 macroconidia by direct injection into the hemocoel, and hemocytic response to the conidia, hyphae, and tissue destruction in the gills of these shrimp was negligible. Perhaps the hemocyte reserve of these shrimp was overwhelmed by an inoculum of that magnitude. In experiments in which fewer macroconidia were injected (Table 1), the time to the onset of mortality decreased, and the challenged shrimp became increasingly able to encapsulate and destroy the macroconidia before germination, thus preventing the death of the shrimp. Encapsulated conidia were not observed to develop hyphae or to show any sign of germination in this study.

Unestam and Weiss (1970) studied the blood reactions in vivo in two species of freshwater crayfish, *Astacus astacus* and *Pacifastacus leniusulus,* to injected zoospores of the pathogenic fungus *Aphanomyces astaci.* Unestam and Nylund (1972) also studied in vitro the reactions of hemocytes from *Astacus astacus* and *P. leniusculus* to hyphae of *Aphanomyces astaci.* In both studies the hemocytes adhered to and encapsulated hyphal elements, and later the hemocyte–hyphae aggregations became melanized. However, the melanization of the hemocyte–spore aggregates and hyphal elements was considerably more pronounced in *P. leniusculus,* which is normally resistant to infection by *Aphanomyces astaci* than in *Astacus astacus* to which *Aphanomyces astaci* is pathogenic.

The findings of the present study, when considered with the studies by Unestam and Weiss (1970) and Unestam and Nylund (1972), indicate that hemocytic activity is an important factor in the apparent resistance of *P. aztecus* and *P. setiferus* to the *Fusarium* sp. that is pathogenic to *P. californiensis.*

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